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Fear or Curiosity: Does a shelter help to be courageous?

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Para a minha mãe, minha sensei, obrigado por tudo e sem ti, eu nunca teria chegado aqui.

Life, will find a way.
- Dr. Ian Malcom

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Resumo

O teste de campo aberto (TCA) teve como objetivo inicial relacionar o número de excrementos num espaço aberto com o índice de timidez do animal, a sua atividade locomotora e exploratória. Atualmente, o número de variáveis usadas neste tipo de teste cresceu substancialmente e as suas características são controladas pelo experimentador e ajustadas às espécies e à questão específica, podendo haver assim, uma elevada variabilidade destas. Para além da componente comportamental, também a avaliação hormonal (através de cortisol ou corticoesterona) se tornou num fator a ser levado em conta no estudo do TCA. Uma das diferenças encontradas entre estudos é a presença e ausência de abrigo. Devido a esta diferença, a interpretação dos resultados obtidos a partir do TCA nem sempre é clara. O objetivo deste trabalho foi de determinar a influência da presença/ausência de um abrigo num TCA na ocorrência de comportamentos relacionados com o stress e níveis hormonais em ratinhos selvagens das espécies *Mus musculus domesticus* e *Mus spretus* capturados nas zonas de Alenquer, Lisboa e serra de Sintra. Esperava-se que com a presença de um abrigo os níveis de stress diminuíssem e atividade exploratória fosse promovida, em especial na espécie *M. spretus*, menos habituada a ambientes humanizados e por isso, mais sensível a situações de stress. Os indivíduos pertencentes ao grupo experimental foram expostos ao TCA com e sem abrigo, e o seu comportamento foi registado durante sessões de 10 minutos de duração para cada tratamento. Após cada sessão foi realizada uma colheita de sangue em cada indivíduo, cujo soro foi analisado em relação aos níveis de concentração de cortisol, os dados foram divididos em três grupos: experimental, controlo e *baseline*. Para a avaliação comportamental, foram escolhidas onze variáveis dependentes: (1) tempo na zona de abrigo/base (2) tempo na zona central, (3) tempo na zona de margem, (4) distância total percorrida, (5) velocidade média, e os comportamentos (6) "ambulation", (7) "rearing", (8) "jump", (9) "grooming", (10) "manipulation" e (11) "freezing". Os resultados mostraram que a presença de um abrigo teve impacto significativo nos níveis hormonais e nas ocorrências comportamentais, sendo o valor de cortisol menor quando um abrigo se encontrava presente em ambas as espécies. A espécie *M. spretus*, foi a que demonstrou maior sensibilidade à presença de um abrigo, pois não apresentou diferenças significativas de valores de cortisol entre o tratamento com abrigo e o grupo de controlo (sem tratamento). Este resultado foi contra o que era esperado pois sendo *M. spretus* uma espécie de ambientes mais naturais, e menos habituada a presença humana, esperava-se que as diferenças de cortisol fossem significativas. Pelo contrário, a espécie *M. m. domesticus*, (uma espécie comensal, e por isso, mais habituada a ambientes e presença humana) apresentou valores de cortisol mais elevados. A nível comportamental, o tempo passado na zona de abrigo/base da arena e comportamentos como "grooming" e "freezing" foram significativamente afetados pela presença/ausência de um abrigo. Os resultados indicam que a configuração do TCA tem um impacto importante nos resultados pelo menos, se a espécie em estudo for selvagem. Espera-se portanto, espera-se que este trabalho possa contribuir para reforçar o papel de testes comportamentais como o

TCA na conservação de espécies ameaçadas, nomeadamente, na seleção de indivíduos para ações de libertação na natureza ou programas de reprodução.

Palavras-chave: Teste de campo aberto, Mus, stress, comportamento, cortisol

Abstract

The open field test (OFT) initially aimed to relate the number of excrements in an open space with the animal's shyness index, its locomotor and exploratory activity. Currently, the number of variables used in this type of test has grown substantially and their characteristics are controlled by the experimenter and adjusted to the species and the specific issue, so there may be a high variability. One of the differences found between published studies is the presence and absence of shelter, because of this difference, the interpretation of the results obtained from the OFT is not always clear. The objective of this work is to determine the influence of the presence / absence of a shelter in an OFT on the occurrence of exploratory and stress-related behaviors and hormone levels in wild *Mus musculus domesticus* and *M. spretus* mice captured in the areas of Alenquer, Lisboa and Serra de Sintra. Individuals in the experimental group were exposed to the OFT configurations with and without a shelter, and had their behavior recorded during 10-minute sessions for each treatment. After each session a blood sample was taken from each individual, whose serum was analyzed for cortisol concentration levels and the data were divided into three groups: experimental, control and baseline. Eleven variables were chosen for the behavioral assessment: (1) time spent in the shelter/base zone (2) time spent in the center zone, (3) time spent at the margin zone, (4) total distance moved, (5) average speed, and (6) ambulation, (7) rearing, (8) jump, (9) grooming, (10) manipulation, and (11) freezing behaviors. The results showed that the presence of a shelter had a significant impact on hormonal levels and behavioral occurrences, with a lower cortisol value when a shelter was present in both species. *M. spretus* showed the highest sensitivity to the presence of a shelter as it did not show significant differences in cortisol values between the shelter treatment and the control group (no treatment). At the behavioral level, the time spent at the center of the arena and behaviors such as grooming, and freezing were significantly affected by the presence / absence of a shelter. The results indicate that the configuration of the OFT has an important impact on the results, at least if the species under study is wild. We hoped this work may contribute to reinforce the role of behavioral testing in the conservation of endangered species, in particular in the selection of individuals for wild release or breeding programs.

Keywords: Mus, behavior, open field test, cortisol, stress

Communications

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*Presenting author

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Introduction

1. The Open Field Test

The Open Field Test (OFT) was created by Hall and Ballechey to test rodent behavior (Hall and Ballechey, 1932; Hall, 1934; Archer, 1973). The initial purpose of this test was to relate the number of droppings in an open field to the animal's shyness (Hall, 1934), its locomotor activity and willingness to explore.

The OFT measures the behaviors that are evoked by placing the animal into a novel and open environment, in which escape is prevented by surrounding walls. The expression of these behaviors depends on several factors, namely, removal from a familiar environment, exposure to a novel environment (experimental arena and surrounding environment), being placed in an open field, and the experiment itself (Carter et al., 2013). The OFT is easy to set up and perform, the data obtained is also simple and easy to analyze. It has thus become a very popular method of measuring activity and exploration of individuals in several other species besides rodents (Carter et al., 2013).

Since Hall's (1934) early work, the number of dependent variables used in the OFT has grown substantially. Not only behaviors (ambulation, freezing, grooming, jumps, manipulation, rearing, etc.) but also autonomic responses of the nervous system (droppings) and adrenal activity (corticosterone) are measured (Blizard et al., 2005).

The characteristics of the arena used in the OFT are controlled by the researcher and adjusted to the species and the hypothesis being tested (Blizard et al., 2003). For example, the size and the shape of the arena can vary, being round, square, rectangular, or even a circular corridor. Further characteristics that can be adjusted are: a) the start of behavioral scoring (immediately, a certain time after the animal has been placed in the arena, or after a certain behavior is observed); b) the duration of the test (predefined duration, or until a certain behavior occurs); c) prior handling; d) start location (center or margin); and e) light intensity (from no light to strong light intensity). Another crucial difference between published studies is the presence of shelter. While some studies offer a shelter in the open field arena (Augustsson and Meyerson, 2004; Augustsson et al., 2005), others don't (Bronikowski et al., 2001; Polissidis, 2017).

Due to all these differences in setup, the interpretation of the results obtained from the OFT is not always unambiguous (Perals et al., 2017). For example, instead of measuring the degree of curiosity and exploration, some authors suggest the OFT measures different types of neophobia (Greggor et al., 2015). Carter and colleagues (2013) also point out that the OFT can measure different traits simultaneously (exploration/curiosity vs. fear/anxiety) depending on the specific experimental settings used. Given the difficulties in interpreting the results obtained from the OFT and the lack of independent validation, some researchers are now becoming more cautious about using this methodology in their research (Reale et al., 2010).

As such, the aim of this thesis is to better understand how changes in the OFT experimental setup affect the outcome of the OFT. Specifically, we will evaluate how the presence/absence of a shelter

influences the exploratory behavior of two mouse species, the expression of stress-related behaviors and their hormonal levels (see *5. Objectives, hypothesis and expected results* for details).

2. The Open Field Test and its use in the conservation of endangered species

Many wild species have been used in the OFT, for example *Microtus rossiaemeridionalis* (Griffin, et al 2000), *Meriones unguiculatus* (Stuermer et al., 2006), *Parus major* (Dingemanse et al., 2002), *Chelonoidis carbonaria* and *Pogona vitticeps* (Moszuti et al., 2017) and *Macaca mulatta* (Hampton et al., 2004). As previously mentioned, the presence or absence of a shelter can have an important influence on the behavior of the individuals in the OFT. This could be even more important in wild species than in laboratory strains. The behavioral traits of wild animals are more strongly connected to natural conditions and some of those traits might even be absent in laboratory animals. It is thus possible that the response of wild species to stressful situations is different from that of laboratory strains (Swart, 2004).

There is also evidence of the existence of behavioral variation at the individual level (Swart, 2004). These consistent behavioral differences between individuals are referred to as animal personality or temperamental traits. They can affect the way individuals interact with their environment, and by this, their survival (Dingemanse, 2003). Some authors thus argue that knowing the individuals' temperament could be a vital tool for improving the success of captive breeding and reintroduction programs (Mcdougall et al., 2006,).

Temperamental differences in fitness-relevant behaviours like anti-predatory, foraging and exploration behaviour can be relevant in the context of conservation (Dingemanse, 2003). A good example of that was observed in *M. rossiaemeridionalis*, in which captive-bred individuals were tested for their mobility levels before being released in the wild. Individuals with higher mobility rates during the tests had lower rates of mortality by predation in the first 3 days after release than those displaying lower mobility rates (Griffin et al., 2000). In this way, the temperament of reintroduced individuals can affect population fitness and by this, the outcome of conservation programs. Still, the influence of animal temperament on conservation programs remains largely unexplored (McDougall et al., 2006).

In captive reproduction programs, the successful pairing of individuals is crucial to maintain a stock of healthy individuals. Additionally, being reintroduction into the wild the ultimate purpose of reproduction programs, it is vital that the selection of individuals for reproduction considers temperamental states that are more beneficial for reintroduction and the survival of released individuals (Griffin et al., 2000).

Temperamental states can be measured by direct observation using tests like the OFT, making it a good tool to help select the more suitable individuals to use in reintroduction and reproduction programs (McDougall et al., 2006). By better understanding how the details of the OFT setup (namely

the presence or absence of a shelter) can influence the animal's motivational state and the test's outcome, one can better use the methodology to help select the most suitable individuals. Considering that the fitness of a species also depends on its ability to explore the environment (e.g., to find food, a partner, or shelter), if the absence of a shelter in the OFT increases this species' stress level, then the majority of tested animals might mainly stay motionless ("freezing" behavior) during the test. If the presence of a shelter decreases the stress levels, there might be room for different types of behaviors, including exploratory ones, to be displayed. In this way, the presence of a shelter would enable the comparison of exploration behavior in the OFT between individuals, facilitating the decision of which individuals are more exploratory and thus possibly more apt to survive and adapt to life in the wild.

The behavior of animals is strongly connected with their hormonal status. For instance, the release of certain hormones during stressful situations is highly associated with the expression of specific behaviors. Among the hormones involved in the stress response are the corticosteroids (cortisol and corticosterone) secreted by the adrenal cortex (Gong et al., 2015). This secretion is increased by stress, which enhances the activity of the hypothalamus-pituitary-adrenal axis (Gong et al., 2015).

This thesis will investigate the influence of a shelter in the OFT on the behavior of two wild mice species, *Mus musculus domesticus* and *M. spretus*. Although, neither of the species has a conservation status, nor is part of a captive breeding or reintroduction program, the findings here obtained may contribute to raise awareness on how simple modifications in methodology, such as the inclusion of a shelter in the OFT, may be especially important when selecting individuals to participate in reproduction and reintroduction programs.

3. The study species

The house mouse (*Mus musculus domesticus*, Linnaeus 1758*)* is a small rodent, with an elongated body (about 90 mm long), developed ears and eyes, a pointed snout, and a long tail (about 95 mm long) (Madureira and Ramalhinho, 1985). It is a commensal species, living near humans, and feeding on their food storages. This intimate association began in the Middle East about 12,000 years ago with the species *M. musculus*, which was exploring the food resources available due to the human presence (Cucchi et al., 2012). Due to this relationship the house mouse has reached a near-global distribution, additionally promoted by human movements and commercial exchanges around the world (Bonhomme and Searle, 2012).

Wild populations of the house mouse occur preferably in humid habitats. Their diet is basically granivorous, although it may include a variety of foods, including products normally consumed by humans. Reproduction can be continuous throughout the year when living commensally, but more reduced when living in uninhabited areas. It is a nocturnal species, having two activity peaks, after sunset and before dawn. These animals are very agile, good swimmers and climbers, being common prey of many carnivores and birds of prey (Madureira and Ramalhinho, 1985).

The Algerian mouse (*Mus spretus*, Lataste 1883) is, in its outer appearance, very similar to *M. m. domesticus* (body length about 85 mm, tail length about 70 mm), but their typical habitat and proximity to humans differs considerably (Madureira and Ramalhinho, 1985). *M. spretus* prefers dry biotopes, occurring in cultivated areas, gardens, vineyards, and grass fields, and usually avoid human habitations (Madureira and Ramalhinho, 1985). Their diet is essentially herbivorous, consuming seeds, stems, leaves and fruits (Madureira and Ramalhinho, 1985). Reproduction in this species appears to be highest in spring and summer. Its activity is essentially nocturnal, with peaks at dawn and dusk (Madureira and Ramalhinho, 1985). It is a a common prey to some carnivores such as foxes (*Vulpes vulpes*), common genets (*Genetta genetta*) and some birds of prey such as the barn-owl (*Tyto alba*) (Madureira and Ramalhinho, 1985).

4. Objectives, hypothesis and expected results

The objective of this study is to determine the influence of the presence of a shelter in an OFT on the expression of stress-related behaviors and hormonal levels in wild mice of the species *M. m. domesticus* and *M. spretus*. For this, mice were exposed to different versions of the OFT (with and without a shelter), their behavior was measured, and their blood cortisol level determined.

Cortisol and corticosterone are closely correlated, and both are often used as biomarkers for stress and depressive disorders (Namakura et al., 1990). In their study, Gong and colleagues (2015) suggested that corticosterone is a more adaptation-related biomarker, while cortisol is rather a quicker responder during severe acute stress. Because in the current study we want to measure short-term stress, we decided to use cortisol as a biomarker. In fact, several studies have observed increased cortisol levels in plasma and adrenal glands of mice following stress (Nakamura et al., 1990), and many others have even used cortisol as an index for stress activation in mice (Zhang et al., 2011).

The main hypothesis is that the presence of a shelter reduces the stress level of animals tested in the OFT, thus increasing their exploratory behaviors (higher distance moved, rearing, speed, manipulation, more time spent in the open area) and decreasing anxiety related behaviors (freezing, jump, wall seeking). The secondary hypothesis is that animals used to human presence have lower stress levels during the OFT, independent of the presence of a shelter.

It is thus expected that (1) both *M. m. domesticus* and *M. spretus* will have lower blood cortisol levels and higher rates of exploratory behavior in OFT in the presence of a shelter than in its absence. It is further expected that (2) *M. spretus* will exhibit a higher level of stress and display a less exploratory behavior than *M. m. domesticus*, since it is a species presumably less accustomed to human presence and artificial environments.

Methods

1. Animal trapping and maintenance

For this study, 32 individuals of *M. m. domesticus* and 33 individuals of *M. spretus* were captured. *M. m. domesticus* were trapped at Quinta da Barreira Branca, Alenquer and Hipódromo do Campo Grande, Lisbon. *M. spretus* were trapped in the Sintra-Cascais Natural Park (Tapada do Saldanha and Tapada do Mouco).

All animals were captured using Sherman and wooden box live traps (Anthony et al., 2005) baited with sardine paste. Hydrophobic cotton placed inside the traps provided thermal insulation. To capture *M. m. domesticus*, the traps were placed in horse stables and animal food storage houses at the end of the day and checked the next morning. Two trapping sessions of 3 to 4 nights each were performed. For *M. spretus*, traps were distributed in habitats typically inhabited by this species in Sintra (small waterways with vegetation and small cliffs or open fields with tall grass). Traps were set at dusk and were active until 1 am. As in those habitats shrews are also present, traps were checked every three hours to avoid any shrews captured accidentally dying from starvation. For *M. spretus*, 4 trapping sessions of 3 to 4 nights were performed.

Captured individuals were transported to the animal facility at the Faculty of Sciences, University of Lisbon. Animals were housed individually in Makrolon type III cages (26.5 x 42.5 x 14 cm) with wood shavings as bedding. Different nesting materials (cotton, hay, toilet paper and cardboard tubes) were used as environmental enrichment. Food (commercial rodent chow safe A04) and water were provided *ad libitum*. Animals were kept under controlled temperature (20 ± 2°C) and light conditions (12L:12D, lights on at 07:00).

Before the beginning of experiments, all individuals underwent a habituation period of one week (week 0); this allowed animals to get acclimated to the laboratory environment, human presence and handling by the experimenter. Habituation to handling was done by taking each individual from its cage once a day and handling the individual for 10 to 15 min. During the habituation period animals were also sexed and weighed. Animals were additionally weighed once a week over the course of experiments to monitor their health condition.

After completion of experiments the tip of the tail was cut from each individual. This was done to obtain biological tissue for genetic analysis to confirm each individual's identity in terms of species and also to ensure that no hybrids were being used (according with Orth et al., 2002, *M. spretus* and *M. m. domesticus* are known to hybridize in nature).

2. Experimental design

Open Field Test

For each species, animals were divided into two groups: a) control group - animals used only for analysis of blood cortisol levels but not in behavioral tests, and b) experimental group - animals used in behavioral tests and analysis of blood cortisol levels. The experimental group for *M. m. domesticus*

was composed by 18 animals, and by 17 animals for *M. spretus*. The control group for *M. m. domesticus* had 12 animals and 11 animals for *M. spretus*. *M. spretus* group was smaller because two animals died during the habituation period. Allocation to a given group was done in a way to keep the sex ratio and origin of individuals balanced among groups.

After the habituation period, individuals from the experimental group were submitted to the open field test (OFT) with a shelter present in the arena (treatment 1) and with no shelter present (i.e., empty arena; treatment 2). To avoid an effect of treatment order, half of the individuals of each group were tested first with treatment 1 and the other half were first tested with treatment 2. This was reversed on the following week, individuals first tested on treatment 1 being then tested on treatment 2 and vice-versa (see Table 1). So, each individual from the experimental group participated twice in the OFT, once with a shelter and once without a shelter present in the arena. Individuals from the control group did not participate in the open field test. Experimental sessions for *M. spretus* took place in March 2018 and in May 2018 for *M. m. domesticus.*

Cortisol

All individuals were sampled for blood (See *4. Blood sample collection* for details) once a week over the course of three weeks, independently of the group they were assigned to (Table 1). On week 1 all individuals were sampled between 10:00 and 12:00h. On weeks 2 and 3, individuals from the experimental group were sampled immediately after the OFT. Individuals from the control group were sampled at a similar time of day. All blood samples taken from a given individual were exactly one week apart.

Blood samples taken during week 1 (no behavioral tests independent of the group) were used to verify if animals from the experimental and control groups had similar baseline levels of cortisol. Blood samples from the animals in the control group (no behavioral tests during all three weeks) were used to assess how blood cortisol levels progressed over time during the course of the experiment (weeks 1-3) and the effect of the experiment itself (control group vs. experimental group on weeks 2 and 3). The influence of a shelter in the OFT was evaluated by comparing blood cortisol levels of animals subjected to the two behavioral treatments (shelter vs. no shelter) on weeks 2 and 3.

Table 1 - Timeline of experimental procedures for *Mus musculus domesticus* and *M. spretus* experimental and control groups. Experimental groups were subdivided into subgroups 1 and 2 according with which treatment was applied first (Shelter vs No shelter). T1- Treatment 1, T2 – Treatment 2, OFT – Open Field Test.

Group / Subgroup		**Week 0**	**Week 1**	**Week 2**	**Week 3**
Control Group		Habituation	Blood collection	Blood collection	Blood collection
Experimental Group	**Subgroup 1**	Habituation	Blood collection	T1: OFT **WITH** shelter Blood collection	T2: OFT **NO** shelter Blood collection
	Subgroup 2	Habituation	Blood collection	T2: OFT **NO** shelter Blood collection	T1: OFT **WITH** shelter Blood collection

3. Experimental setup and procedure

Open Field Test

The experimental setup consisted of a square terrarium (80 cm x 80 cm, height 65 cm – Figure 1). The terrarium was lined with wooden panels on the sides and with a thin matt-white plastic sheet on the base. Lining of the terrarium was necessary to make the arena walls opaque and also to eliminate reflections during the recording of experiments.

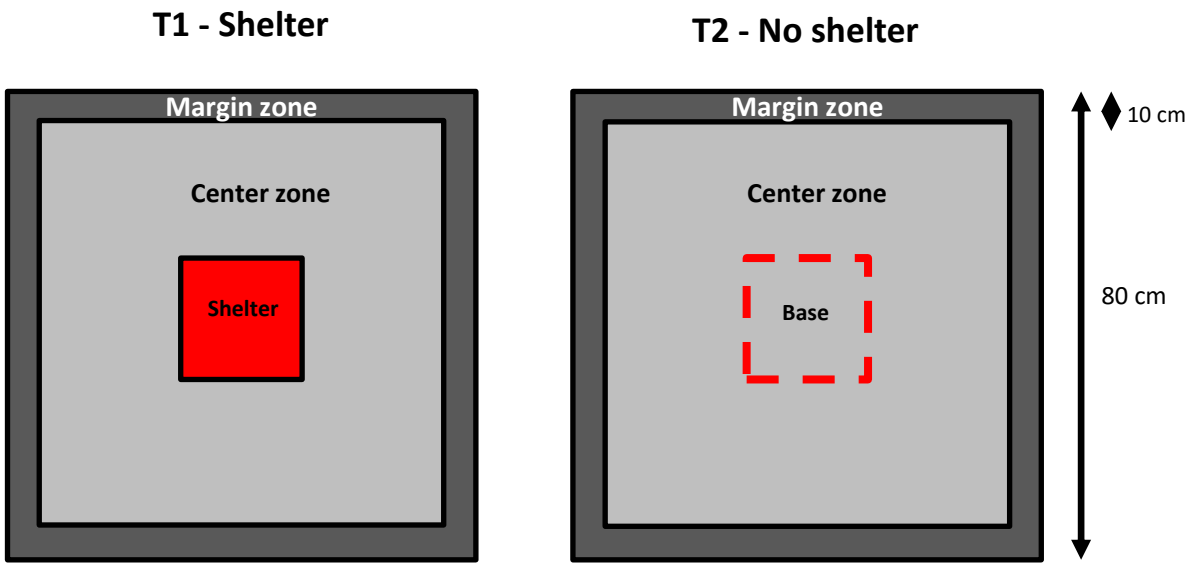

Figure 1 - Schematic view of the OFT arena for each treatment (T1 - with shelter, T2- without shelter). The three zones (margin zone, center zone and shelter/base zone) were defined in the software ANY-maze and used in the analysis of animal positioning.

Depending on the treatment, a shelter or a base was placed in the center of the arena. The base consisted of a 10 x 10 cm white plastic square; the shelter consisted on the same base but covered with a transparent red plastic half tube (height: 3.6 cm, length: 7.5 cm; Figure 2A).

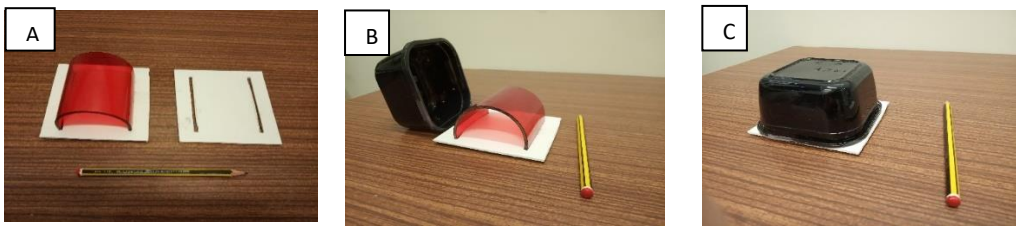

Figure 2 - Shelter, base and black starting cover used in the OFT. (A) Shelter on top of base (left), and base without shelter (right). (B) Shelter and starting cover. (C) Shelter covered with starting cover.

At the start of each test, the test individual was placed inside the shelter/on top of the base, covered with the black plastic cover (Figure 2C) and placed in the center of the arena. After a habituation period of one minute the cover was taken off. The test began as soon as the animal left the shelter/base and had a duration of 10 min. The order in which each animal was tested in each treatment (shelter and no shelter) was assigned randomly, with an interval of one week between the tests (Table 1).

All tests were recorded with a video camera (Canon Legria HF S200), placed above the arena, under white light (fluorescent lamp). The experimenter was absent from the experimental room during the experiment.

After each test the test animal was retrieved from the arena, the number of droppings was counted, and the arena was cleaned with an alcohol solution (70 %) to remove any chemical cues that could influence the behavior of the following test animal.

The videos were analyzed with two types of software, the video-tracking software ANY-MAZE video tracking system version 4.56h Beta (Stoelting, U.S.A.) and the event-recorder software BORIS (Torino, Italy). ANY-MAZE was used to analyze the individuals' movements in the OFT. This software allowed to digitally divide the arena in three zones: Margin zone, Center zone and Shelter/Base zone (Figure 1), and calculate total time spent in each zone, distance travelled and average speed. BORIS was used to determine the number of events of the most common behaviors observed during the OFT: *Ambulation*, *Freezing*, *Grooming*, *Jump*, *Manipulation* and *Rearing* (see Table 2).

Table 2 – Behavioral elements scored during the Open Field Test for *Mus musculus domesticus* and *M. spretus.*

Behavior	Description of behavior
Ambulation	The animal moves in any direction with possible changes in direction and speed. During ambulation the animal might perform exploratory movements of the head while sniffing the air. Short stops (less than 3 s) may occur, in which the animal raises the head and sometimes one or both front legs (see *Rearing* behavior). The duration of this behavior varies greatly but was only recorded when it had a duration of at least 3 s.
Freezing	The animal is completely motionless; no movement, including head or nose, is visible. The duration of this behavior varies greatly but was only recorded when it had a duration of least 3 s.
Grooming	The animal cleans itself following a typical sequence; the animal starts by using its paws to clean the head, licking the hair in the ventral and lateral parts of the body, and licking the tail from the base to its tip. This behavior is often repeated several times.
Jump	The animal jumps against the wall or any other place in the arena. Isolated jumps or multi-jump sequences may occur.
Manipulation	The animal scratches or nibbles at parts of the arena, shelter or shelter base, or performs digging-like behaviors. These movements are very fast and short and are often performed together or in quick succession.
Rearing	The animal stands on the two hind legs acquiring a vertical posture using the tail as support on the ground. When rearing is performed near one of the walls of the arena, the animal often uses the front legs to support the weight of the body on the wall of the arena. This behavior may occur during *ambulation* but was only recorded when it had a minimum duration of 3 s.

Cortisol

Three blood samples were collected from each individual. Individuals were anesthetized with Isoflurane (van Zupthen et al., 2001) and blood was collected using the retro-orbital sinus technique. For this, a capillary is used to pierce the lacrimal membrane in the edge of the eye and puncture the orbital sinus in a way that the blood flows through the capillary (Kumar et al., 2017). To avoid causing eye injuries or other related health issues to the individuals, blood samples were taken alternately from both eyes, i.e., in successive weeks different eyes were used.

Blood samples were collected in Eppendorf tubes and left to coagulate for one hour at ambient temperature. After that they were placed in a centrifuge and spun for 20 minutes at 2.000 G to separate the blood serum from solid blood cells. The serum was then stored at -80ºC until further analysis.

Cortisol levels in the serum were analyzed using the BioVision "Cortisol (human/mouse/rat) ELISA Kit", following the guidelines of the kit's protocol (See appendix I). This kit is an in vitro assay for the quantitative measurement of cortisol in serum and plasma.

4. Statistical analyses

All statistical analyses were made using the software IBM SPSS Statistics (version 24). Dependent variables were tested for normality and the statistical test chosen accordingly: paired t-tests and repeated ANOVA measures for normally distributed variables, paired Mann-Whitney U tests and repeated measures Kruskal-Wallis tests for not normally distributed variables.

No statistical differences in cortisol levels were found between weeks 2 and 3 for the control group (no behavioral tests) in either species (see Results section). As such, to analyze the effect of the treatment, the data from weeks 2 and 3 of the experimental groups was pooled according to treatment: *Shelter* vs. *No shelter*. The data of the control group was used as treatment "*No test*".

Open Field Test

The aim of the analysis of the behavioral data was to test the influence of a shelter in the behavior of the two mouse species in an OFT. For this, eleven dependent variables were chosen. Five variables were related with the position, movement and speed of the test animal: (1) total time spent in the shelter/base zone, (2) total time spent in the center zone, (3) total time spent in the margin zone, (4) total distance moved, (5) average speed (for definition of zones, see Figure 1). The remaining six variables concerned the number of events of specific behavioral elements: (6) *Ambulation*, (7) *Freezing*, (8) *Grooming*, (9) *Jump*, (10) *Manipulation*, and (11) *Rearing* (Figure 1, Table 2). These variables were analyzed for each species separately, using Student's t-tests and Mann-Whitney U tests (depending on the distribution of the respective dependent variable) with treatment (*Shelter* vs. *No shelter*) as the predictor variable. To analyze the behavioral data in terms of species differences, the respective tests (Student's t-test and Mann-Whitney U test) were performed on the same dependent variables using species as the predictor variable.

Cortisol

Data for cortisol levels was divided into three datasets: baseline (i.e., cortisol values obtained during week 1 for experimental and control groups), experimental and control datasets.

Baseline dataset – comparison of cortisol levels in week 1

A Kruskal-Wallis test was done for each species to compare the baseline cortisol concentrations between groups (control group, experimental subgroup 1, experimental subgroup 2) before the experiment. Cortisol concentrations served as a dependent variable and group as a predictor variable. To analyze pairwise differences between groups, posthoc Dunn-Bonferroni tests were

performed. The data used were the cortisol concentrations obtained from the baseline measurements (i.e., blood collections in week 1).

Control dataset – comparison of cortisol levels of the control group over the three weeks

A Kruskal-Wallis test was made for *M. m. domesticus* and an ANOVA for *M. spretus* to compare the cortisol concentrations over the course of the experiment (weeks 1-3). Cortisol concentrations served as a dependent variable and week as a predictor variable. To analyze pairwise differences between weeks, the respective posthoc tests were performed (Dunn-Bonferroni tests for *M. m. domesticus* and Tukey HSD tests for *M. spretus*). The data used were the cortisol concentrations obtained during all three weeks from animals of the control group (i.e., animals that did not participate in any behavioral tests).

Experimental and control datasets – comparison between the two treatments and control

An ANOVA was used to compare the cortisol concentrations between the *Shelter* treatment, the *No shelter* treatment and the *No test* treatment (i.e., control) for *M. m. domesticus* and a Kruskal-Wallis test for *M. spretus*. Cortisol concentrations served as a dependent variable and treatment (*Shelter*, *No shelter* and *No test*) as a predictor variable. To analyze pairwise differences between the treatments, the respective posthoc tests were performed (Dunn-Bonferroni tests for *M. spretus* and Tukey HSD tests for *M. m. domesticus*). The data used were the cortisol concentrations obtained during the two experimental weeks (week 2 and 3) from all animals.

Experimental dataset – comparison between species separated by treatment

A Mann-Whitney and Student's t-tests were used to compare the cortisol concentration between the two species (Mann - Whitney test) for *Shelter* treatment, (Student's t-test) for *No shelter* treatment and (Mann – Whitney test) for *No test* treatment (i.e. control). Cortisol concentrations served as a dependent variable and species as a predictor variable. The data used were the cortisol concentrations obtained weeks 2 and 3.

5. Ethical note

All experimental procedures were approved by Orbea, the animal welfare body of the Faculty of Sciences, University of Lisbon (Statement number 1/2018). Trapping, transport and maintenance of captured animals was authorized by the Portuguese authority for the conservation of nature and forests, "Instituto de Conservação da Natureza e Florestas" (license number 428/2018). All females identified as being pregnant or lactating were immediately released at the capture location. After completion of experiments, *M. spretus* were released at the locations where they had been captured; *M. m. domesticus* were euthanized following the ethical guidelines for the use of animals in behavioral research (Buchanan et al., 2012).

Results

1. Open Field Test

Shelter vs. No Shelter vs. Control

M. m. domesticus spent significantly more time in the shelter/base zone when a shelter was present (U = 211.000, p = 0.001, N = 32, Figure 3A) and in the margin zone when a shelter was absent (t = 0.059, p =0.000, N = 32, Figure 3B). Time spent in the center zone (U = 166.000, p = 0.160, N = 32, Figure 3C), total distance moved (t = 1.085, p = 0.829, N = 32, Figure 3D) and mean speed (t = 1.104, p = 0.837, N = 32, Figure 3E) did not differ significantly between treatments (i.e., *Shelter* vs. *No shelter*).

Similarly, *M. spretus* spent significantly more time in the shelter/base zone when a shelter was present (U = 195.500, p =0.031, N = 33, Figure 3A), but did not spend a significantly different amount of time in the margin zone (U = 103.000, p = 0.245, N = 33; Figure 3B). Time spent in the center zone (t = 0.160, p = 0.473, N = 33, Figure 3C), total distance moved (t = 0.162, p = 0.732, N = 33; Figure 3D) and mean speed (t = 0.162, p = 0.737, N = 33; Figure 3E) did also not differ significantly between treatments in this species.

M. m. domesticus showed a significantly higher number of *Freezing* events in the absence of a shelter (U = 63.000, p = 0.014; N = 32; Figure 4A). However, none of the remaining behavioral parameters recorded were significantly different between treatments: *Ambulation* (t = 0.753, p = 0.411, N = 32; Figure 4B) *Grooming* (U = 138.000, p = 0.724, N = 32; Figure 4C), *Jump* (t = 0.074, p = 0.754 N = 32; Figure 4D), *Manipulation* (U = 126.000, p = 0.956, N = 32; Figure 4E), and *Rearing* (t = 1.058, p = 0.502, N = 32; Figure 4F).

In *M. spretus* none of the recorded behavioral parameters was significantly different between treatments: *Ambulation* (U= 128.000, p = 0.790, N = 33, Figure 4B), *Freezing* (U= 113.000, p = 0.423, N = 33, Figure 4A), *Grooming* (t = 1.241, p = 0.915, N = 33, Figure 4C) *Jump* (U= 116.000, p = 0.488, N = 33, Figure 4D), *Manipulation* (U = 143.000, p = 0.817, N = 33, Figure 4E), *Rearing* (t = 1.704, p = 0.962, N = 33, Figure 4F).

Species comparison

Grooming events were significantly more common in *M. spretus* than in *M. m. domesticus* (t = 12.992, p < 0.001, N = 65; Figure 4C), while *Ambulation* (t = 2.014, p = 0.034, N = 65; Figure 4B) and *Jump* (U = 891.500, p < 0.001, N = 65; Figure 4D) were significantly more common in *M. m. domesticus.* There were no significant differences between species in the remaining behavioral parameters analyzed: *Freezing* (U = 418.000, p = 0.120, N = 65; Figure 4A), *Manipulation* (U = 441.000, p = 0.234, N = 65; Figure 4E), *Rearing* (t (63) = 4.822, p = 0.676 N = 65; Figure 4F), total distance moved (t = 0.070, p = 0.876, N = 65; Figure 3A), and mean speed (t = 0.073, p = 0.875, N =

65; Figure 3E). Also, the time spent at the Shelter/base zone (U= 665.000, p = 0.072; N = 65; Figure 3A), center zone (F (63) = 0.365, p = 0.259, N = 65; Figure 3C) and margin zone (t = 0.001, p = 0.558, N = 65; Figure 3B) was not significantly different between species.

2. Cortisol

Baseline dataset – comparison of cortisol levels in week 1

There were no significant differences between the three groups (control group, experimental subgroup 1, experimental subgroup 2) in baseline cortisol levels for both *M. spretus* (H (2) = 0.157, p = 0.925, N = 9) and *M. m. domesticus* (H (2) = 0.766, p = 0.682, N = 8).

Control dataset – comparison of cortisol levels of the control group over the three weeks

In both species, cortisol concentrations differed significantly between weeks (*M. spretus*: F (2) = 4.372, p = 0.035, N = 16; *M. m. domesticus*: H (2) = 6.054, p = 0.048, N= 17; Figure 5). In both species this resulted from lower cortisol concentrations in week 3 as opposed to week 1 (*M. m. domesticus*: p = 0.042; *M. spretus*: p = 0.012). There were no significant differences between weeks 1 and 2 and between weeks 2 and 3 in either species (*M. m. domesticus* - week 1 vs. week 2: p = 0.618, week 2 vs. week 3: p = 0.841; *M. spretus* - week 1 vs. week 2: p = 0.088, week 2 vs. week 3: p = 0.322).

Experimental and control dataset – comparison between the two treatments and control

Cortisol levels differed significantly between treatments (*Shelter*, *No shelter*, *No test*) in both species (*M. spretus*: H (2) = 16.069, p < 0.001; N = 16; *M. m. domesticus*: F (2) = 21.409, p < 0.001; N = 17 Figure 6). *M. spretus* had significantly higher cortisol levels in the *No shelter* treatment as opposed to the *No Test* (p < 0.001) and the *Shelter* treatment (p = 0.011). *M. m. domesticus* had significantly higher cortisol levels in both the *Shelter* and the *No shelter* treatment as opposed to the *No test* treatment (*Shelter* vs. *No test*: p < 0.001; *No shelter* vs. *No test*: p < 0.001).

Experimental dataset – comparison between species separated by treatment

When a shelter was present in the OFT, *M. spretus* had significantly lower cortisol levels than *M. m. domesticus* (U = 59.000, p = 0.003; N = 16; Figure 6). However, in the absence of a shelter, both species showed similarly high cortisol levels (t (11) = 9.041, p = 0.289; N = 16; Figure 6). In the control groups, both species showed similarly low levels of cortisol (U = 15.000, p = 0.152; N = 15; Figure 6).

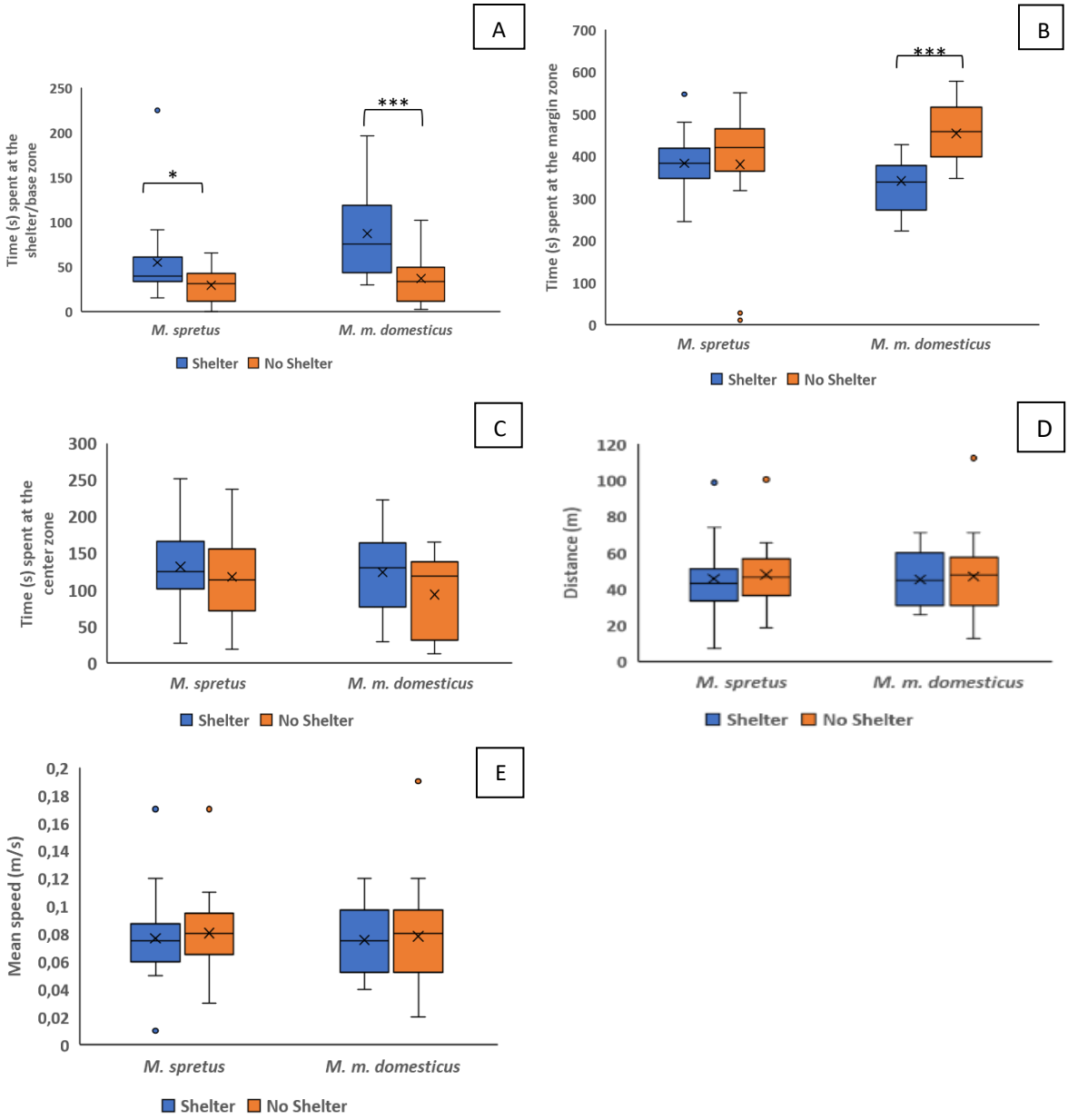

Figure 3 - Open field test results for *Mus musculus domesticus* and *M. spretus* in the presence (blue) and absence of a shelter (orange). (A) total time spent in the shelter/base zone (s), (B) total time spent in the margin zone (s), (C) total time spent in the center zone (s) (D) total distance moved (m), and (E) mean speed (m/s),. Significance levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Boxes denote the second and third quartile range, whiskered lines the 95% confidence intervals, dots the outliers, X the mean, and the line the median.

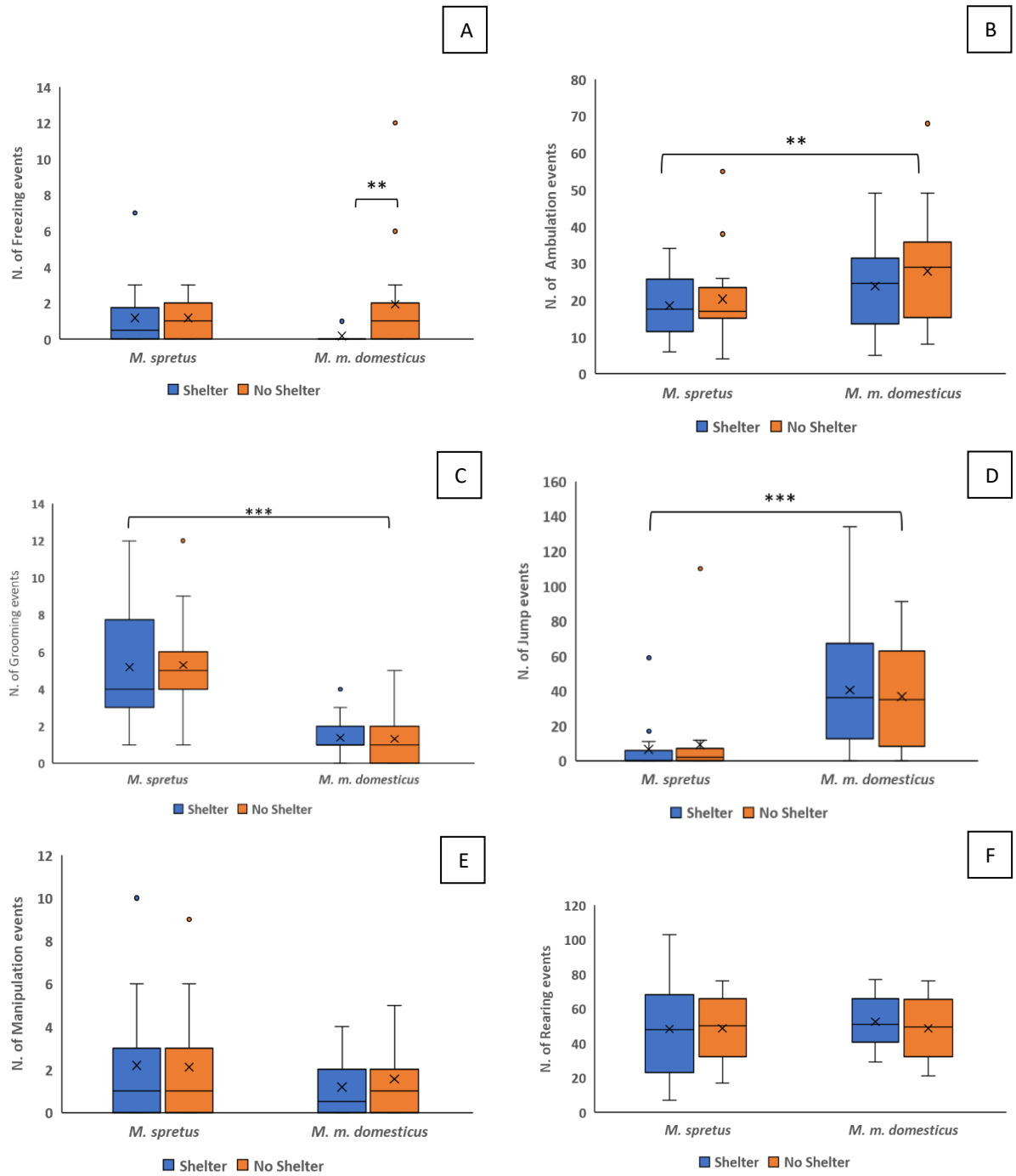

Figure 4 - Open field test results for *Mus musculus domesticus* and *M. spretus* in the presence (blue) and absence of a shelter (orange). Number of events recorded for each behavioral element: (A) *freezing*, (B) *ambulation*, (C) *grooming*, (D) *jump*, (E) *manipulation* and (F) *rearing*. Significance levels: * signifies $P \leq 0.05$, ** signifies $P \leq 0.01$, *** signifies $P \leq 0.001$. Boxes denote the second and third quartile range, whiskered lines the 95% confidence intervals, dots the outliers, X the mean, and the line the median.

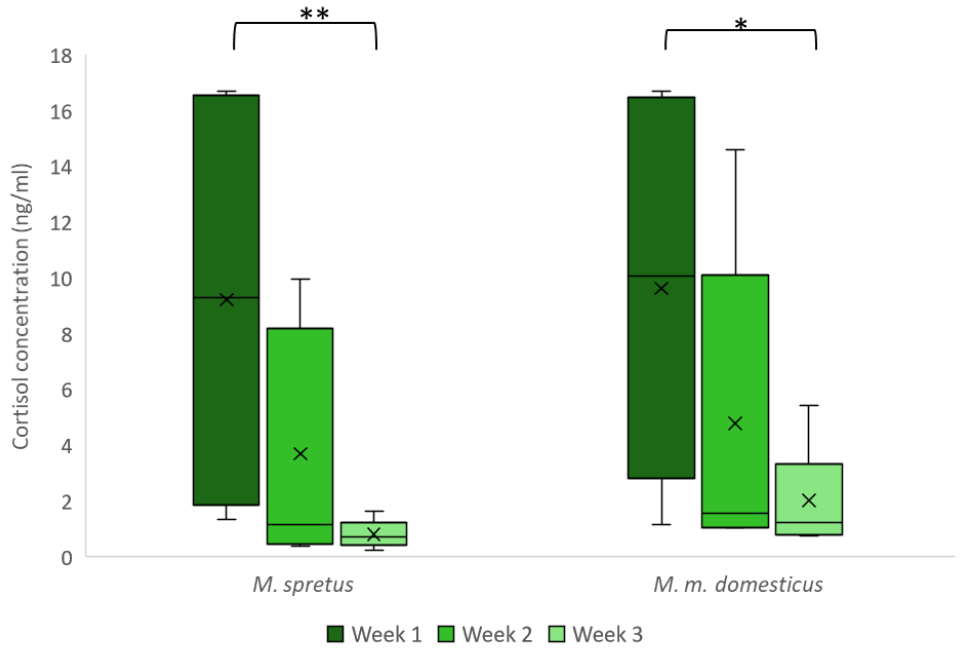

Figure 5 - Cortisol levels of *Mus spretus* and *M. musculus domesticus*. Comparison along the three weeks of the control groups (no OFT). Significance levels: * $p \leq 0.05$, ** $p \leq 0.01$. Boxes denote the second and third quartile range, whiskered lines the 95% confidence intervals, dots the outliers, X the mean, and the line the median.

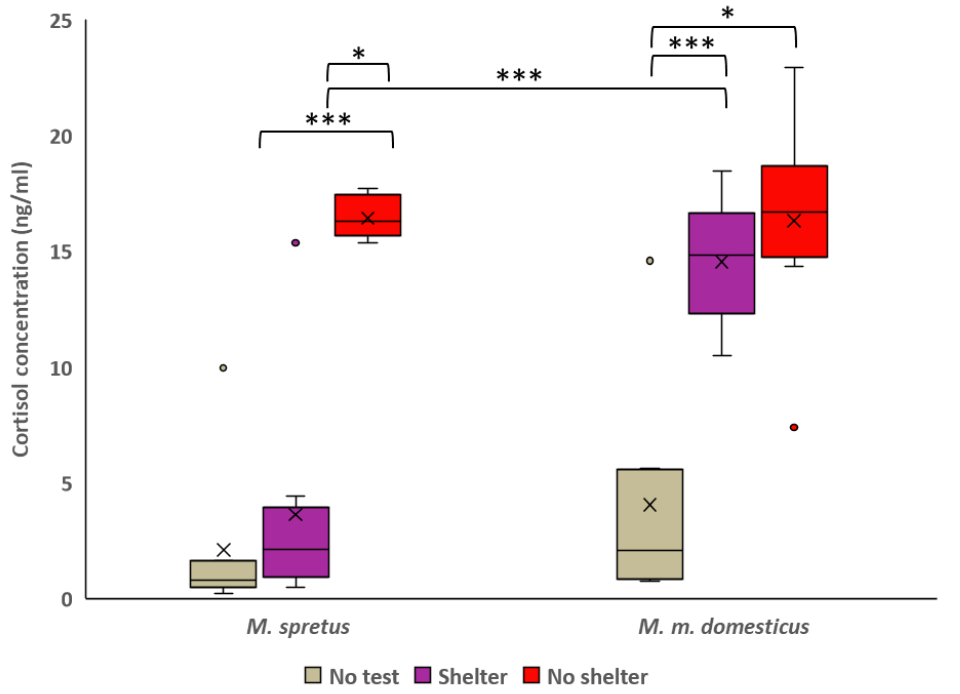

Figure 6 - Cortisol levels of *Mus spretus* (experimental groups and control group) and *M. musculus domesticus* (experimental group and control group). Comparison of the three treatments (*No test*, *Shelter*, *No shelter*). Significance levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Boxes denote the second and third quartile range, whiskered lines the 95% confidence intervals, dots the outliers, X the mean, and the line the median.

Discussion

The objective of this study was to determine the impact of a shelter on the stress levels and the behavior of animals exposed to an open field test (OFT). The main hypothesis was that the presence of a shelter in an OFT, would induce lower the cortisol levels in two mouse species (*M. m. domesticus* and *M. spretus*) and display a more exploratory behavior, in contrast to an OFT without a shelter. The cortisol levels were indeed lower for both species (statistically significant only in *M. spretus*) when the shelter was present. However, contrary to what was predicted, the presence of a shelter did not increase the exploratory behavior of either mouse species.

The secondary hypothesis was that the commensal species (*M. m. domesticus*) would present lower stress levels during the OFT, independent if there was a shelter present or not. However, the results obtained were the opposite of what was expected: *M. m. domesticus* always showed higher levels of stress during the OFT than *M. spretus*.

1. Mouse stress levels in the open field test

Influence of the shelter on stress levels

There were significant differences in cortisol levels between the two treatments for *M. spretus*. In the presence of a shelter, *M. spretus* had lower cortisol levels than in its absence. In fact, when a shelter was present, cortisol levels were not significantly different to those of the control group. This suggests that when a shelter is present in the OFT, *M. spretus* might experience similarly low levels of stress as to not experiencing the OFT at all. A probable explanation for this is that *M. spretus* experiences the shelter as a safe place, its presence in the arena being sufficient to counteract the stress caused by being placed in an open space such as the OFT arena. As opposed to *M. spretus*, the presence of a shelter in the OFT did not change the cortisol levels of *M. m. domesticus*, which were similarly high both in the presence as well as in the absence of a shelter.

M. m. domesticus thus showed higher levels of cortisol than *M. spretus* in the presence of a shelter. This was an unexpected result considering that *M. m. domesticus* is a commensal species (Vujanic et al., 2011; Cucchi et al., 2012), and as such should show a greater tolerance to stress caused by novel environments and proximity to humans. However, it was *M. spretus*, which tends to inhabit more natural habitats and at a greater distance to human environments (Madureira and Ramalhinho, 1985, Fons et al., 1988), that demonstrated lower levels of cortisol in the presence of a shelter. It is difficult to explain this result. Possibly in *M. m. domesticus* the cortisol released in reaction to novel situations is naturally higher than in *M. spretus*. Several authors (Yin et al., 2007, Gong et al., 2015) found that when laboratory mice were exposed to unpredictable sources of stress for the first time, they showed very high cortisol levels. The maximum levels reached in those studies are in the range of those observed in the current study in *M. m. domesticus* when exposed to the OFT. The mentioned studies also showed no significant decrease of cortisol levels when laboratory mice were repeatedly exposed to stressors in subsequent days.

Current laboratory mice are the result of more than 100 years of selective breeding of mainly *M. m. domesticus* individuals. Despite only representing a very limited amount of the genetic diversity found in wild mouse populations (Yang et al., 2011), laboratory strains still maintain many of the characteristics found in wild individuals. As such, it is reasonable to expect that similarly to laboratory mice, the wild *M. m. domesticus* used in our study respond in a similar manner, showing relatively strong stress manifestations when exposed to novel and stressful situations.

Ganem (1991) found physiological differences between commensal and outdoor populations of *M. m. domesticus*. Commensal populations living in animal farms, displayed higher levels of plasma corticosterone when compared to an outdoor population living along the Mediterranean coast. According to this study, differences in plasma corticosterone might be a way for mice to adapt to their social environment. As opposed to outdoor mice, commensal mice are commonly exposed to higher levels of social stress due to higher population densities. Commensal mice might thus respond to added stress by maintaining higher levels of baseline plasma corticosterone, low enough to avoid secondary effects, but sufficiently high to permit these individuals to respond quickly to changes in their environment.

Temporal changes of stress levels

The level of cortisol in each species' control group decreased over the three-week period of blood sampling. The most likely explanation for this pattern is that, as intended, the animals were habituating to the experimental procedures (i.e., handling, anesthesia and blood collection). These individuals had already been subjected to the same handling protocol during the habituation period (week 0), which possibly further promoted their habituation to the laboratory and experimental procedures. Also Gong and colleagues (2015) noticed that in rats, cortisol hormone levels would increase to its highest level on the first day of unpredictable stress and decreased on the following days. This type of habituation has also been observed in studies on different species of zoo animals. When trained to participate voluntarily in medical actions (blood collection, vaccinations, etc.), individuals manage to stay calm and lower their stress levels, as they already know the procedure they will be subjected to, appearing to be in control of the situation (Mineka et al., 1986; Veissier, 2007). Another possible explanation for this pattern, which may occur along with the above hypothesis, is that, as the researchers gained practice collecting blood and the procedure was performed more efficiently, less stress was experienced by the animals.

The fact that cortisol levels continued to decrease after the habituation week in both species' control groups raises the question if it would have been more appropriate to begin experiments only in week 3. Cortisol levels were indeed lower in the third week than in the second week. They were, however, not significantly different, suggesting that even though animals were possibly not fully habituated to laboratory conditions and procedures in week 2, their stress levels were reasonably lower and should not have influenced the results obtained in a significant manner.

Levels of blood cortisol in mice follow a daily pattern. Several authors (Kim et al., 2008, Dickmeis 2009, Gong et al., 2015) have found that mice have the lowest levels of cortisol between 8.00 and 15.00 h. Our experiments were conducted within this time period (between 10:00 and 12:00 h), which is appropriate, as any kind of increase should be observable and would not be covered by a natural daily fluctuation. Therefore, the high levels obtained during the OFT without a shelter are most probably caused by the absence of a shelter and not by daily fluctuations in hormonal patterns.

2. Behavior of mice in the open field test

Influence of a shelter on mouse behavior

Several behavioral differences were found in both species between the OFT with and without shelter. Like expected, both species spent more time in the shelter zone when a shelter was present. It is a natural part of mice behavior to preferentially stay hidden, especially in wild species. Augustsson and Meyerson (2004) had observed that wild mice had a higher tendency to seek shelter than laboratory strains. On the contrary, in the absence of a shelter, *M. m. domesticus* spent more time in the margin zone, were they most likely experienced a higher feeling of safety.

It is well known that rodents tend to stay close to walls and objects, avoiding open areas, a behavior commonly referred to as wall-seeking (Hughes, 1994, Plesner et al., 2003). The higher amount of wall-seeking observed in *M. m. domesticus* might be related to the fact that they had higher levels of cortisol than *M. spretus*, so they were possibly experiencing more stress. With the presence of a shelter, however, a safe point was created in the normally most exposed area of the arena (Pennycuik, 1987), which gave both species the opportunity to hide in a location more safe than close to the wall. The higher amount of wall-seeking observed in *M. m. domesticus* might be related to the fact that they had higher levels of cortisol than *M. spretus*, so they were possibly experiencing more stress. With the presence of a shelter, however, a safe point was created in the normally most exposed area of the arena (Pennycuik, 1987), which gave both species the opportunity to hide in a location more safe than close to the wall.

M. m. domesticus showed fewer freezing events in the presence of a shelter, a behavior commonly associated to stress, thus suggesting that the shelter helps reduce *M. m. domesticus* stress levels and potentially has an impact on the results of the OFT. However, *M. m. domesticus* cortisol levels do not seem to support this interpretation; cortisol levels of *M. m. domesticus* were similarly high both in the presence and absence of a shelter. A possible explanation for this could be related with the amount of time animals spent in the open field (outside the shelter). When a shelter was present *M. m. domesticus* stayed long periods of time hidden inside the shelter, thus spending considerably less time in the remaining open areas of the arena. As such, it is possible that *M. m. domesticus* displayed a smaller number of freezing events because it also spent considerably less time in the open. Alternatively, a similar number of freezing events might have occurred during the OFT, however, because *M. m.*

domesticus was inside the shelter these were unaccounted for during the behavioral analysis. A more detailed analysis of *M. m. domesticus* behavior should help clarify this.

None of the species spent more time in the center zone when a shelter was present in the OFT. Thus, in contrast to our predictions, the presence of a shelter did not increase the exploratory behavior of either mouse species. A similar result was found by Augustsson and Meyersson (2004). In their study, wild mice showed a clear avoidance of open areas, a tendency to seek shelter and a highly cautious strategy during exploration.

Species differences in mouse behavior

When comparing the two species behavior in the OFT, *M. m. domesticus* showed higher rates of ambulation and jumping events. In their study with *M. m. domesticus* and *M. m. musculus*, Vošlajerová-Bímová and colleagues (2016), also found that commensal species spend more time exploring an unfamiliar environment, compared to non-commensal ones. Also, Frynta and others (2018) showed a high number of ambulation and climbing events for *M. m. domesticus*, attributing this to boldness. According to Mota (1987) high ambulation scores are usually seen as indicative of reduced fear in animals and active exploration of the environment. However, in the current study the commensal species, *M. m. domesticus*, had higher levels of cortisol than the non-commensal species, *M. spretus*. So, possibly, *M. m. domesticus* might experience more fear in novel situations, but are more adapted to deal with it, seeking escape or novel resources actively. The high levels of jump and ambulation behavior recorded in *M. m. domesticus* would thus fit to the high cortisol levels recorded in this species (Yin et al., 2007, Gong et al., 2015).

M. spretus displayed significantly more instances of grooming behavior than *M. m domesticus*. Grooming serves many purposes (Spruijt, 1992), such as cleaning (Amador and Hu, 2015), thermoregulation (Almeida et al., 2015), pain relief (Spradley et al., 2012), social interaction (Carter and Wilkinson, 2015), and stress and anxiety responses (Molesti and Majolo, 2013). Spruijt and colleagues (1992) defined a way of distinguishing between grooming for body care and grooming due to stressful situations (conflict grooming), based on the length of each grooming bout and the completeness of its sequence. According to them, conflict grooming mainly occurs in short bouts and may therefore only include grooming of the head regions. Body care is a behavior of lower priority that only occurs when the animal is relaxed. It occurs in long bouts and complete sequences that include licking the whole body. Like in their study, the grooming behavior observed in the present study, was manifested through long bouts and sequences (personal observation, not analyzed statistically). It may thus be interpreted as a calm response after an excitatory stimulus that was overcome, not a direct result of the excitement per se (Füzesi et al., 2016), in accordance with the lower levels of cortisol recorded for *M. spretus* in this study.

Possible applications of this study

It is often argued that the OFT presents the animals with an excessively simplified and artificial environment (excessively open, brightly illuminated, and monotonous). According to them, this artificial environment does not promote manifestations of species natural behavior, nor does it provide a demonstration of normal anxiety levels (Genaro and Schmidek, 2000). Considering this, some authors proposed to replace the OFT by observations of the animal's behavior directly in the cages where the animals are kept (Stanford, 2007). Stanford (2007) also argues that the period of the day observations are made can also have an influence on the results, and proposes to restrict observations to the night period, thus taking into account that certain species, like mice, are nocturnal or crepuscular. Another important issue to take into account, is the fact that animals under OFT conditions, are observed in isolation; for social animals, such as rats and mice, isolation serves as a powerful stressogenic factor, significantly influencing their behavior (Stanford, 2007). All the factors mentioned above might even have a more significant effect in wild species, and thus have a greater influence in the experimental outcome. For example, considering that the study species are both nocturnal/crepuscular, the obtained results could have been different if the observations and blood collection had been made during the night period; the cortisol levels and occurrences of stress behaviors like freezing might have been lower, since the animals would have been tested during their normal activity period, an issue that could be verified in future studies. The social isolation is an important factor to take in account. Considering that mice are social animals, the fact that they were kept and tested in isolation, might have increased their stress levels. It would be important to conduct a study under similar conditions but with animals kept and tested in social groups and compare the results with those of the present study.

3. Final Conclusions

The main results from this study were, 1) contrary to the predictions, the presence of a shelter did not promote exploratory behavior in either species; 2) there were behavioral differences depending on the presence of a shelter (time spent in the shelter or the margin, respectively); 3) there were behavioral differences between species (more jump and ambulation events in *M. m. domesticus*, and more grooming events in *M. spretus*), 4) both species presented significant differences in cortisol concentrations during the OFT when a shelter was present (higher in *M. m. domesticus*); 5) the presence of a shelter significantly reduced the stress levels of *M. spretus*, but not of *M. m. domesticus*.

In this study we focused on the influence a shelter in the OFT has in the exploratory behavior of mice. Since several other variables are usually manipulated in the OFT (the presence of light, light intensity, shape of the arena, etc.), it would be important to test the impact each of these variables on the outcome of OFT in future studies.

The continuous decrease in cortisol levels in the control group suggests that possibly only one week of habituation is not enough to get complete normal values of cortisol. It might be advisable to provide 2 weeks of habituation before the start of the behavioral test.

The OFT may be a useful tool in species conservation when used to test certain behavioral traits and thus help select individuals more suitable for breeding and/or reintroduction programs (Griffin et al., 2000; Festa-Bianchet and Apollonio, 2003, McDougall et al., 2006). *M. spretus* could potentially be used in future studies as a rodent model to understand the best way to use the OFT for conservation purposes given its presence in more naturalized habitats (Madureira and Ramalhinho, 1985, Fons et al. 1988) compared to *M. m. domesticus*. As such, their behavior in the OFT could be used as a reference for other wild rodent species. When a shelter was present, *M. spretus* cortisol levels were not significantly different from those of control individuals, even though they were exposed to a new artificial environment (the OFT arena). That is, in the presence of a shelter, animals were less stressed, and displayed a possibly more natural behavior. This suggests that, if the OFT was used to select individuals more apt to be released in the wild, it would be important to offer a shelter in the OFT to promote a more natural behavioral state, to allow a better prediction of how the animals might behave in the wild.

We hope our results may contribute to increase the link between conservation actions and animal behavior research through the application of this type of methodologies in real situations of conservation or reproduction in captivity projects of endangered species.

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Appendix

FOR RESEARCH USE ONLY!

BioVision
BioVision Incorporated

Cortisol (human/mouse/rat) ELISA Kit

1/15

(Catalog # K7430-100, 100 assays; Refer to Section V for Storage)

I. Introduction:

BioVision's Cortisol Enzyme-Linked Immunosorbent Assay (ELISA) Kit is an in vitro assay for the quantitative measurement of Cortisol in serum and plasma. Cortisol is a steroid hormone released from the adrenal cortex in response to the hormone ACTH. It increases blood pressure and blood sugar levels, and suppresses the immune system. Cortisol acts through specific intracellular receptors and has effects on numerous physiologic systems, including immune system, glucose-counter regulation, vascular tone, substrate utilization and bone metabolism. It exists in the blood as either a free form or bound to corticosteroid-binding globulin (CBG). The amount of Cortisol present in serum undergoes diurnal variation, with the highest levels present in the early morning and lower levels at night. BioVision's Cortisol ELISA kit is a competitive enzyme immunoassay kit. The assay employs an antibody specific for cortisol coated on a 96-well plate. Cortisol in a sample or a HRP molecule which has Cortisol covalently attached to it is bound to the wells by immobilized antibody in a competitive manner. After a simultaneous incubation, the excess reagents are washed and a TMB substrate is added to the wells. Color develops in proportion to the amount of bound Cortisol HRP conjugate, and the intensity is inversely proportional to the concentration of Cortisol in the sample. Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm. Sensitivity of the kit is 1 ng/ml and detection range is from 5 ng/ml to 480 ng/ml. The cross-reactivity of this ELISA assay is 6.8% for prednisolone, 4.22% for cortisone, and <0.1% for other hormones, including 11-deoxycortisol, estradiol, testosterone, and progesterone. The intra-assay reproducibility as measured by the coefficient of variation (CV) is < 8 % & inter-assay has CV < 12 %.

II. Application:

Quantitative measurement of Cortisol

III. Specificity:

Human, mouse, rat

IV. Sample Type:

- Serum and plasma
- Cell culture supernatant

V. Kit Contents:

Components	K7430-100	Cap Code	Part No.	Storage Temp.
Plate Coated with Cortisol Ab	12 stripsx8 wells	-	K7430-100-1	-20°C
Assay Diluent	15 ml	WM	K7430-100-2	4°C
Wash Buffer (10x)	20 ml	NM/Brown	K7430-100-3	4°C
Standard Diluent	15 ml	NM	K7430-100-4	4°C
Cortisol Standard (4800 ng/ml)	0.5 ml	Yellow	K7430-100-5	-20°C
Cortisol HRP Conjugate (2000x)	8 µl	Green	K7430-100-6	4°C
TMB Substrate	11 ml	Amber	K7430-100-7	4°C
Stop Solution	11 ml	NM/Blue	K7430-100-8	4°C
Plate Sealer	2	-	K7430-100-9	RT/4°C

VI. User Supplied Reagents and Equipment:

- Microplate reader capable of measuring absorbance at 450 nm.
- Absorbent paper.
- Distilled or deionized water.

VII. Storage Conditions and Reagent Preparation:

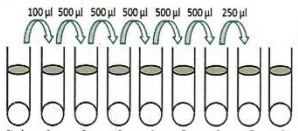
Kit can be used within one year if stored according to storage instructions. Avoid repeated freeze-thaw cycles. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay. Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

- **Wash Buffer (10x):** Dilute with deionized or distilled water to a final working 1x buffer concentration. If the Wash Buffer (10x) contains visible crystals, warm to room temperature and mix gently until dissolved before dilution.
- **Cortisol HRP Conjugate (2000x):** Dilute to 1x in Assay Diluent. Dilute only the necessary amount of Cortisol HRP Conjugate just before use. Recommendation: Dilute necessary amount of Cortisol HRP Conjugate 10 fold in 1x Wash Buffer. Further dilute this solution 200 fold in Assay Diluent to the 1x concentration.

VIII. Assay Protocol:

1. Bring all Buffers and desired number of Ab coated strips to room temperature (18 - 25°C) before use. It is recommended to run all Standard dilutions in duplicate.
2. Prepare a series of dilutions for Cortisol Standard (4800 ng/ml) in Standard Diluent as shown in Figure 1. Mix each tube gently and thoroughly before the next transfer. Standard Diluent alone serves as the zero Standard (0 ng/ml). **Note:** Discard unused diluted Standard solution.
3. Pipette 20 µl of Cortisol Standards or sample into their respective wells.
4. Add 100 µl of 1x Cortisol HRP Conjugate/well into appropriate wells. Cover

Figure 1



Tube	Stock	1	2	3	4	5	6	7	8
Standard Diluent (µl)	-	900	500	500	500	500	500	500	500
Final Conc. (ng/ml)		480	240	120	60	30	15	5	0

Biovision cortisol ELISA kit protocol page 1

wells with Plate Sealer and incubate with gentle shaking for 1-2 min. at room temperature. Incubate for 1 hr at 37°C.

5. Discard the solution. Wash 4 times (each wash for 3-4 min.) with 200 µl 1x Wash Buffer with gentle shaking.
6. Add 100 µl of TMB Substrate/well and gently shake. Measure absorbance at 650 nm for 1-4 min. at room temperature to monitor the blue color development, intensity of which is inversely proportional to the concentration of cortisol in samples and Standards.

   Notes:

   a. Incubation time after addition of TMB substrate can be optimized to avoid over-development of color. Recommended absorbance is ~0.8-1.2 at 650 nm.

   b. Optional: Prepare one parallel well for background control and add TMB Substrate.
7. Add 100 µl of Stop Solution into each well including background control and mix with gentle shaking. Remove air bubbles if any. Read at 450 nm within 5 min.
8. **Calculation:** Calculate the mean absorbance for each set of duplicate Standards. Plot Cortisol Standard Curve. Calculate Cortisol concentration of sample by interpolation of the Standard Curve. If sample was diluted, multiply the value by dilution factor to calculate the concentration of Cortisol in the sample.

   Note: Background subtraction for each reading is optional for calculating the sample Cortisol concentration, and will not change the final results.

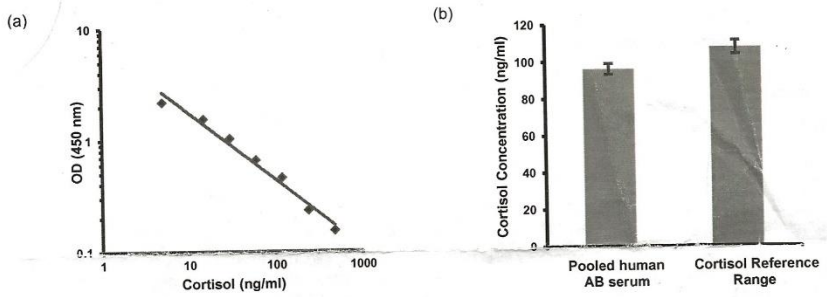


Figure 2: a) Cortisol Standard Curve. This standard curve is for demonstration only. A standard curve must be run with each assay. b) Measurement of Cortisol concentration in human serum. Sample of pooled human serum (20 µl) was analyzed following the kit protocol (left) and compared to the literature values (right).

IX. RELATED PRODUCTS:

Adrenocorticotropic Hormone (ACTH) (human) ELISA Kit (K4003-100)
Prolactin (human) ELISA Kit (K4687-100)
Prolactin (mouse/rat) ELISA Kit (K4688-100)
Growth Hormone (human) ELISA Kit (K7412-100)
Luteinizing Hormone [LH] (human) ELISA Kit (K7426-100)
Thyroid Stimulating Hormone (human) ELISA Kit (K7411-100)

FOR RESEARCH USE ONLY! Not to be used on humans.

Biovision cortisol ELISA kit protocol page 2